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Molecular Characterization of Chloramphenicol-Resistant *Haemophilus parainfluenzae* and *Haemophilus ducreyi*

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We examined chloramphenicol-resistant *Haemophilus parainfluenzae* and *Haemophilus ducreyi* strains isolated in various parts of the world. The antibiotic resistance determinants were located on conjugative plasmids in *H. ducreyi*, but were chromosomally located in *H. parainfluenzae*. Both species produced chloramphenicol acetyltransferases (CATs) that were sensitive to 5,5'-dithiobis(2-nitrobenzoic acid) like the enteric type II and *Haemophilus influenzae* CAT enzymes, but differed from these enzymes in elution patterns and subunit molecular weight. Southern blot analysis showed the *H. parainfluenzae* and *H. ducreyi* CAT genes were molecularly related to the enteric type II class as well as the *H. influenzae* CAT. Heterogeneity of the physiochemical properties of the CATs was observed; however, the data suggested that all three *Haemophilus* spp. have a common ancestral source for the CATs.

In a relatively short time the *Haemophilus* spp. population, previously regarded as uniformly susceptible, has become resistant to a number of antibiotics, including ampicillin (4, 8, 15, 26), streptomycin (7), kanamycin (7), tetracycline (2, 10, 13, 15), chloramphenicol (9, 10, 15, 20, 21, 23, 27), and sulfonamides (1). In most cases resistance has been plasmid mediated due to genes related or identical to those mediated by R factors in the *Enterobacteriaceae* (1, 4, 8, 11, 15, 18).

Recently, we have characterized plasmid-mediated chloramphenicol acetyltransferases (CATs) isolated from chloramphenicol-resistant (Cm^r) *Haemophilus influenzae* (18). The enzymes had properties in common with the gram-negative family of CATs; however, instead of being similar to the most widespread variant type I, these appeared to belong to the less common type II group (18).

In this report we examine the genetic and biochemical basis of chloramphenicol resistance in *Haemophilus parainfluenzae* and *Haemophilus ducreyi*. Both species were resistant due to the production of CATs. The genes encoding the enzymes appeared to be located on the *H. parainfluenzae* chromosome, whereas the CAT genes from *H. ducreyi* were located on conjugative R plasmids. Both groups of enzymes had some characteristics in common with the type II class of CAT, but also had novel physiochemical properties.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains are listed in Table 1. The solid medium used for growth of *H. parainfluenzae* was modified GC agar (Difco Laboratories, Detroit, Mich.) (19, 20). The medium for *H. ducreyi* was modified GC agar supplemented with 5% heated sheep blood and 0.026% cysteine (chocolate agar) (26). For certain experiments the solid medium was supplemented with various concentrations of chloramphenicol. Supplemented 3.5% brain heart infusion (Difco) was used to grow *H. parainfluenzae* and *Escherichia coli* (19). Plates were grown at 36.5°C in 5% Co_2, whereas liquid cultures were incubated at 37°C.

Determination of MICs. The MICs were determined by agar dilutions with a Steers replicator. An inoculum of 10^5 CFU was used (20, 24).

Preparation of labeled plasmid DNA and agarose gel electrophoresis. Cleared lysates of the *H. ducreyi* and *H. parainfluenzae* strains were prepared and electrophoresed through a 0.7% agarose gel (9, 16, 20). The molecular mass of each plasmid was calculated as previously described (16, 20). [^{3}H]thymidine-labeled pRI234 from *H. influenzae* strain HC234 and unlabeled and plasmid DNA were prepared as previously described (9, 20).

Cloning the CAT genes. Samples of purified plasmid DNA were completely digested with the *Eco*RI and *Hin*dIII or *Pst*I and cloned into the vector pBR322. Ligation reactions were carried out at 15°C for 12 h. An approximate 1:1 ratio of vector ends to target DNA was used. T4 DNA ligase was prepared as described by Tait et al. (25). The transformation of *E. coli* HB101 cells with the ligation mixture was carried out as previously described (6). Transformed *E. coli* HB101 cells were selected on agar plates supplemented with 20 μg of chloramphenicol per ml. The clones were then screened by gel electrophoresis.

DNA-DNA hybridization and Southern blots. Unlabeled whole cell DNA from the various strains was prepared (20). Hybridization was done at 65°C for 18 h in 0.21 M NaCl and assayed by the S1 endonuclease technique, using in vivo-labeled plasmid DNA (5, 9, 20). In situ filter blot hybridization was performed with nick-translated, radiolabeled cloned fragments (28) from pRI234 and pMR385 (*H. influenzae*) and Sa (enteric type II CAT) with Gene Screen Plus as described by the manufacturer (65°C without formamide; New England Nuclear Corp., Boston, Mass.). Probes were prepared by running restricted plasmid DNA through a 0.7% agarose gel, excising the desired band, and removing the agarose by the freeze-thaw method (10).

Restriction enzymes. All enzymes were purchased from Pharmacia P-L Biochemicals (Piscataway, N.J.) and used according to the manufacturer's specifications.

Preparation, purification, and assay of CAT. Crude cell

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TABLE 1. Bacterial strains and plasmids

Strain	Year of isolation	Geographic area	Site of isolation	Plasmid[a] (Mdal) or description	Resistance[b]	References
H. ducreyi						
V1159	1979	United States	Chancroid	7.4, 34	Cm^r Tc^r Ap^r	26
V1169	1979	Philippines	Chancroid	7.4, 34	Cm^r Tc^r Ap^r	26
134	1982	Singapore	Chancroid	7.4, 34	Cm^r Tc^r Ap^r Km^r	This study
H. parainfluenzae						
UB2832	1975	England	Pharynx	2.7, 0.75	Cm^r Tc^r	9
79-23164	1979	Kuala Lumpur	Urethra	7.4	Cm^r Tc^r	15
H. influenzae						
HC234	1976	Netherlands	Pharynx	38(pRI234)	Cm^r Tc^r	20
R385	1979	United States	Cerebrospinal fluid	38(pMR385)	Cm^r Tc^r Ap^r	20
Rd	1944	United States	Pharynx		Rif^r Str^r Ery^r	20
E. coli						
HB101(pBR325)				3.7	Cm^r (type I) Ap^r Tc^r	3
J53(Sa)				23	Cm^r (type II) Km^r Sm^r Su^r	20
J53(R387)				41	Cm^r (type III)	20
HB101(pJHC-A18)				*Eco*RI-*Hin*dIII 1.4-kb fragment from pRI234 cloned into pBR322		This study
HB101(pJHC-A17)				*Eco*RI-*Hin*dIII 1.4-kb fragment from pMR385 cloned onto pBR322		This study
HB101(pJHC-A34)				*Pst*I 2.7-kb fragment from R387 cloned onto pBR322		This study

[a] The 7.4-Mdal plasmids encode Ap^r and the 34-Mdal plasmids determine Cm^r and Tc^r. The 2.7- and 0.75-Mdal plasmids are cryptic (9).
[b] Plasmid-mediated resistance: Ap^r, ampicillin; Cm^r, chloramphenicol; Km^r, kanamycin; Sm^r, streptomycin; Su^r, sulfonamide; Tc^r, tetracycline. Chromosomal resistance: Ery^r, erythromycin; Rif^r, rifampin: Str, streptomycin. Plasmids Sa and R387 carry protoype II and type III CAT (22).

lysates were prepared by sonic disruption and stored at −20°C. The enzymatic acetylation of chloramphenicol was followed spectrophotometrically (18, 23). All enzymes were purified from crude extracts by affinity chromatography with a highly substituted Sepharose support (12, 18, 22, 29). The elution profile was determined by washing the Sepharose with 0.3 M NaCl and then with 0.3 M NaCl plus 5 mM Cm, followed by 0.6 M NaCl and 0.6 M NaCl plus 5 mM Cm and finally 1.0 M NaCl. Each enzyme was run on polyacrylamide gel to assess purity; a single band was seen for each.

Inhibition of CAT by DTNB and immunological studies. The crude lysates were tested for sensitivity to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a reagent which modifies reactive thiol groups. Type II enzymes are generally sensitive to DTNB, whereas type I and III enzymes are resistant (18). Ouchterlony diffusion tests were performed with type I, II, and III antisera and purified enzymes (12, 18).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Apparent subunit molecular weights of CAT variants were determined by electrophoresis through a 12% polyacrylamide gel (14). The type I, II, and III CATs were used as molecular weight markers (18).

Mating experiments. Filter matings were performed with recipient strain *H. influenzae* Rd (rifampin resistant [Rif^r], erythromycin resistant [Ery^r], and streptomycin resistant [Str^r]). Matings with *H. ducreyi* were performed directly on chocolate agar plates (19).

Transformation. DNA was prepared from the *H. parainfluenzae* strains as previously described (20). Transformation experiments were carried out with an Rd Rif^r Ery^r Str^r strain as the recipient, and selection was on agar containing 10 μg of chloramphenicol and 10 μg of rifampin per ml. Transformation frequency was determined by the number of transformants divided by the total number of bacteria plated. The phenotype of the transformed colonies was confirmed by checking the strains for resistance to erythromycin and streptomycin (18).

RESULTS

Location of chloramphenicol resistance genes. Cleared lysates were electrophoresed on a 0.7% agarose gel. *H. ducreyi* strains V1159, V1169, and 134 each carried two plasmid species of 7.4 and 34 megadaltons (Mdal). The 7.4-Mdal plasmids pUW115 (from V1159), and pUW117 (from pUW1169) have previously been characterized and shown to carry the gene for TEM-1 β-lactamase (26). Similarly the 7.4-Mdal plasmid from strain 134 carried the gene for TEM β-lactamase and was highly related to the other two plasmids (data not shown). *H. parainfluenzae* strain 79-23164 carried a 7.4-Mdal plasmid that encoded ampicillin resistance and was highly related to the *H. ducreyi* ampicillin plasmids (data not shown). Strain UB2832 has previously been shown to carry two small cryptic plasmids, which was reconfirmed on our gels (9).

Matings were performed to determine whether chloramphenicol resistance was transferable between strains. All three *H. ducrecyi* strains were capable of transferring chloramphenicol resistance at a frequency of 10^{-5} to 10^{-6} per recipient. In previous studies with *H. influenzae* plasmids, Tc^r was transferred with Cm^r in 75 to 100% of the resulting transformants (20). Similar cotransfer was found when the *H. ducreyi* plasmids were examined. In each case both resistant markers were associated with the transfer of the 34-Mdal plasmid. In some experiments ampillicin resistance was also transferred. This was correlated with the presence of the 7.4-Mdal plasmid in the transconjugants. Neither *H. parainfluenzae* strain was able to transfer antibiotic resistances to recipient strains by conjugation, but resistance could be transmitted by transformation. The frequency of transfer was 10^{-6} to 10^{-7} per recipient and was generally 10-fold lower than transfer of the chromosomal markers (Rif^r, Ery^r, or methionine) and 10-fold higher than plasmid transformation. Ninety percent of the Cm^r transformants were also Tc^r. Shearing reduced the linkage between the two determinants, as did treating the donor DNA preparation with restriction endonucleases. However with both types of treated DNA we could still obtain Cm^r transformants.

Various laboratories have shown a high degree of DNA sequences in common between various large R plasmids isolated from *H. influenzae*, regardless of what resistance genes they encoded (2, 9, 11, 13, 20). Therefore, it was of interest to determine whether the 34-Mdal plasmids from *H. ducreyi* were members of the *H. influenzae* family of plasmids and whether these core DNA sequences also existed in *H. parainfluenzae*. *H. influenzae* plasmid pRI234, which encodes both Cm^r and Tc^r, was chosen (9, 20). This plasmid carries both determinants on a transposon-like structure that has designated Tn*1894* (11). Tn*1894* represents 21 to 23% of the entire plasmid and shares no DNA homology with the smaller, nonconjugative ampicillin plasmids described for *H. ducreyi*, *H. influenzae*, and *H. parainfluenzae* (9). The DNA sequences of pRI234, outside of the transposon, have been shown to be highly related to a variety of other plasmids and are representative of this plasmid family (9, 20).

The *H. ducreyi* plasmids shared 70 to 80% of their sequences in common with pRI234. This is considerably more than can be accounted for by the common resistance determinants in the probe. In contrast, the *H. parainfluenzae* strains shared only 20 to 21% homology with the probe, a percentage similar to the proportion of sequences represented by the transposon Tn*1894* (Table 2).

TABLE 2. Hybridization between ^3H-labeled pRI234 plasmid DNA and unlabeled whole cell DNA

Strain[a]	Relative DNA sequence homology with plasmid DNA[b] (%)
HC234	100
V1159	77
V1169	84
134	70
UB2832	21
79-23164	20

[a] Source of unlabeled whole cell DNA.

[b] The actual extent of binding of ^3H-labeled pRI234 with whole cell DNA of the parent (HC234) was 80%. All other reactions were normalized to this value, taken as 100%.

TABLE 3. Characteristics of CATs

Strain	Chloramphenicol MIC[a] (μg/ml)	Molecular wt	Rate of inhibition of DTNB[b] (*K*)	Molarity of NaCl[c] required for elution from affinity resin
H. ducreyi				
V1159	30	24,450	0.044	0.3
V1169	30	24,500	0.022	0.3
134	20	24,650	0.057	0.3
H. parainfluenzae				
UB2832	40	24,750	0.037	0.3
79-23164	50	24,150	0.028	0.3
E. coli				
J53(Sa)		24,150	0.055	0.6
HB101(pJHC-A18)		24,500	0.048	0.6
HB101(pJHC-A34)		25,000	0.007	0.3

[a] Determined by agar dilution.

[b] The pseudo-first-order rate constant (*K*) for the inactivation of enzymes by DTNB was calculated graphically from the equation $\ln E/E_0 = -Kt$, where E/E_0 is the fraction of initial activity remaining after treatment for time t.

[c] The CATs from strain V1159, V1169, 134, UB2832, and 79-23164 required 5 mM chloramphenicol for elution, whereas the type II and III CATs and the cloned pRI234 did not require chloramphenicol.

Characterization of CATs. Crude extracts were prepared from each strain. All of the extracts were capable of acetylating chloramphenicol only when acetyl coenzyme A was present. This suggested that each strain was producing a CAT-like enzyme. Similar CAT activity was produced when the cells were grown in both the presence and absence of chloramphenicol. The MICs of cells exposed to chloramphenicol were identical to those of cells that were not exposed, indicating that the enzymes were produced constitutively (Table 3).

The *H. influenzae* CATs and the majority of other type II enzymes are sensitive to DTNB, a thiol reagent used in the CAT assay. In contrast, the type I and III CATs are resistant to this reagent (18, 19, 23). All five *Haemophilus* spp. enzymes in this study showed inactivation in the presence of DTNB. The rate of inactivation was similar to the type II CAT from plasmid Sa and the cloned *H. influenzae* enzymes (Table 3).

Affinity chromatography was used to purify the various enzymes. Crude extracts were run through a resin column. Greater than 99% of the measurable activity was bound, and 80 to 100% of the measurable activity was recovered for all strains. The enteric type II CAT from plasmid Sa and the *H. influenzae* CAT cloned from plasmid pRI234 (pJHC-A18) remained bound when 0.3 M NaCl and 5 mM chloramphenicol were added to the column and required 0.6 M NaCl for elution. In contrast, the enteric type III CAT, cloned from R385, eluted when 0.3 M NaCl without chloramphenicol was added to the column (Table 3). The *H. parainfluenzae* and *H. ducreyi* enzymes had an intermediate affinity for the Sepharose column and came off when 0.3 M NaCl plus 5 mM chloramphenicol was added, but not in 0.3 M NaCl.

Antisera raised against enteric type I, II, and III CATs were tested in double-diffusion gels. No precipitin bands were formed when any of the purified *Haemophilus* spp. enzymes were tested. The Sa enzyme reacted with the type II antiserum, but not with type I or III antiserum. Similarly,

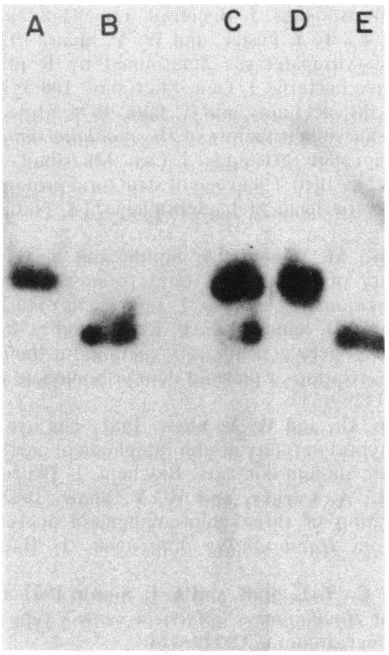

FIG. 1. Southern blot of *Puv*II-digested whole cell DNA against radiolabeled *Eco*RI-*Hin*dIII fragment of pJHC-A18 carrying the *H. influenzae* CAT gene. Lanes: A, *H. influenzae* HC234; B, *H. parainfluenzae* 79-23164; C, *H. ducreyi* V1169; D, *H. ducreyi* 134; E, *H. parainfluenzae* UB2832.

the other antisera formed bands with the homologous cloned enzyme, but not with the other two classes.

The apparent molecular weights of the enzymes were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All five had subunit molecular weights between 24,000 and 25,000 daltons (Table 3).

Genetic relationship among CAT regions. Previously we have shown that DNA fragments encoding the type I, II, or III CATs do not hybridize with each other when used as probes against Southern blots (L. A. Actis, M. C. Roberts, and J. H. Crosa, manuscript in preparation). The cloned *H. influenzae* CATs hybridized with the type II enzyme represented by Sa, but not the type I or III genes (Actis et al., in preparation). Therefore we examined the genes encoding the *H. parainfluenzae* and *H. ducreyi* CAT genes by using Southern blots probed with type II CAT, from Sa, cloned *Haemophilus* spp. CAT genes, and the cloned type I and III CAT genes. The *H. influenzae* CAT (pJHC-A18 and pJHC-A19) showed no homology with the plasmids from *H. parainfluenzae*, but homology was found in the *H. parainfluenzae* chromosomal band and in the 34-Mdal *H. ducreyi* plasmids. Restricted DNA from *H. influenzae* HC234, the three *H. ducreyi* strains, and the *H. parainfluenzae* strains reacted with the type II CAT and both cloned CAT probes from *H. influenzae*. Similar-sized DNA fragments from the *H. influenzae* and *H. ducreyi* strains reacted with the probe, whereas smaller DNA fragments hybridized with the DNA from the two *H. parainfluenzae* strains (Fig. 1). In contrast, type I and III DNA probes did not hybridize with any of the *Haemophilus* spp. DNA.

DISCUSSION

In this paper we examined chloramphenicol resistance in five *H. parainfluenzae* and *H. ducreyi* strains isolated over 7 years in different geographical areas. We have shown that both the chloramphenicol and tetracycline resistance genes are located on conjugative R plasmids in *H. ducreyi*. These plasmids share 70 to 80% of their DNA sequences in common with *H. influenzae* plasmid pRI234. This homology can not be accounted for by the resistance determinants alone, but must extend into the core DNA region of the plasmid. Albritton et al. (2) found similar restriction patterns among three *H. ducreyi* R factors encoding tetracycline resistance and R factors from *H. influenzae*. However, they made no attempt to determine whether the DNA sequences were similar. Our data, which examined the DNA sequences of three different plasmids, support the suggestion that the large conjugative R factors from *H. ducreyi* are molecularly related to previously described R factors in *H. influenzae*.

In contrast, the *H. parainfluenzae* strains could not transfer resistance by conjugation and shared only 20 to 21% of their DNA sequences with the probe plasmid. This homology could be accounted for by the sequences represented by Tn*1894* (11) and suggests that no DNA from the core region of the conjugative R plasmid family is present in these two strains. Similarly Southern blot analysis showed no hybridization between the CAT gene and the *H. parainfluenzae* plasmids as visualized by agarose gel, nor was any homology seen when whole plasmid pRI234 was used as a probe (unpublished observations). Transformation studies enabled us to show that the Cm^r and Tc^r genes in *H. parainfluenzae* could be transferred into a susceptible recipient in a linked fashion. Linkage was reduced when the donor DNA was sheared or treated with restriction enzymes; however, Cm^r transformants were still obtained. Together the data suggest that these genes are located on the *H. parainfluenzae* chromosome. However, it is still possible that the Cm^r and Tc^r determinants are located on nontransmissible plasmids that migrated with the chromosomal DNA. Similar locations have also been described for Tc^r determinants in a few *H. influenzae* and *H. ducreyi* strains (15).

CAT enzymes were characterized and purified from each strain. Some of the properties were similar to the type II enteric CAT, from plasmid Sa, and the *H. influenzae* CAT enzymes, whereas others were novel. To examine the genetic relationship among these strains, Southern blots were performed. All five strains hybridized with the type II and cloned *H. influenzae* probes. This confirms earlier hybridization data, which showed 15 to 20% DNA homology between the enteric plasmid Sa and the *H. influenzae* plasmid pRI234 (unpublished observations). This level of homology could be accounted for by the DNA sequences encoding the CAT gene. In contrast, neither the type I nor the type III CAT gene hybridized. Therefore, this suggests an ancestral relationship between all of the *Haemophilus* spp. genes and the type II enzyme class represented by Sa. One other CAT from a Paris *H. parainfluenzae* strain has been characterized (23). The location of this CAT gene was unclear because no plasmid was identified in the cleared lysate. However the Paris strain was able to transfer both chloramphenicol and tetracycline resistance by conjugation, whereas our two *H. parainfluenzae* strains could not. The CAT isolated from the Paris *H. parainfluenzae* strain had characteristics remarkably similar to those of our five *Haemophilus* spp. enzymes. Shaw et al. (23) concluded on the basis of N-terminal amino acid sequence analysis that the CAT enzyme isolated from the Paris *H. parainfluenzae* strain was most likely related to the type II CAT class, even though some of the physiochemical properties were different than those of the classical type II enzyme. Unfortunately, the original isolate is no longer available, but the transconjugant was supplied by

W. V. Shaw. The DNA from the *E. coli* transconjugant did hybridize with the type II, suggesting that it also is related to the *Haemophilus* spp. enzymes characterized in this study.

We have now examined 100 Cm^r strains from three *Haemophilus* spp.; all carry constitutively produced Tc^r determinants, which hybridize with Tn*10* and are linked to a type II CAT; most are found on conjugative plasmids. Whether this is due to a single event that introduced this combination into the genus or whether multiple events with a similar gene combination occurred is not clear. However, the data suggest that these strains carry related genes encoding proteins with slightly altered physical characteristics as compared with the enteric CAT encoded by plasmid Sa and also from the previously characterized *H. influenzae* CAT enzymes.

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